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THE OPERATION OF THE BACTERIOLOGICAL LABORATORY FOR DAIRY PLANTS

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INTRODUCTION

THE LAST QUARTER-CENTURY has witnessed a vast improvement in methods of milk production. The milk of today is a relatively safe staple. Yet no longer ago than 1900, milk was anything but safe. Methods of production were bad; little control was attempted. Gradually, in city after city, the quality of milk has been improved. Greater and greater proportions of the country's supply have been subjected to a rigorous control until today there is little excuse for a low-grade product.

Bacteria have long been used as a measure of milk quality. It is through their agency that milk becomes unwholesome or at times unsafe. They cause the milk distributor endless worry. They can and do develop odors and flavors. They can and do cause disease. They are an index to what a milk really is. Good milk, by and large, has fewer bacteria; and good milk is produced only through vigilance.

Milk distributors, realizing this fact, frequently pay a premium for milk produced at a predetermined bacterial content. The maximum number of bacteria permitted in any such premium system is purely an agreement between buyer and seller. Though entirely countenanced by regulatory bodies, it is not codified in any laws. Under such system one cannot discriminate between two men producing equal grades of milk. Thus if a premium is rightfully paid to one person, any other person is equally entitled to a similar payment for a similar grade of milk.

To determine the bacterial numbers for such premium payment requires certain laboratory procedures; and since these are somewhat complex and require skill, only competent operators should perform them.

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This reasoning leads directly to the conclusion that operators should be licensed. Under the California law:

> Any person desiring to secure a license to make bacteriological determinations upon milk or cream which determinations are to be used as a basis of payment or determining value.....shall make application in writing on an appropriate form supplied by the Department of Agriculture of the State of California. Before a technician's license is issued to such applicant he shall be required to pass a written, oral and laboratory examination and demonstration of technique sufficiently thorough to indicate the ability of the applicant in making bacteriological determinations on milk and cream which are reasonably accurate, and secure in each of these examinations a grade of at least seventy per cent.
>
> The technique followed in determining the bacterial content of milk or cream shall be in a manner described in the current edition of the *Standard Methods of Milk Analysis*[2] of the American Public Health Association.

The cost of the license is $5.00, with an annual renewal fee of $1.00.

Standard Methods of Milk Analysis gives carefully drawn rules defining each step in the entire process of plating milk. It also carefully defines the microscopic technique; nothing is omitted. Unfortunately, however, these rules, presuppose some knowledge of bacteriology; the directions are somewhat difficult for those unversed in bacteriological methods.

This circular will enlarge on these standard methods—will explain and describe where explanation and description are lacking. It is written more for the untrained man, since regulatory officials presumably have this experience. The official *Standard Methods of Milk Analysis,* not this circular, should of course be the final guide.

Standard Methods describes and recommends three different procedures: (1) the plate count, (2) the direct count (a microscopic method), and (3) the methylene blue reduction test. Of these three methods the plate count is most important, or at least most used. Although most of the advance in milk sanitation has undoubtedly been made through the plate count, all three methods have their place. The bacteriologist should not depend upon any one of them; each tells its story. One supplements the other, and a better knowledge of the milk results. If dependence were placed on the plate method alone, valuable information might be lacking. In the first place, not all bacteria can grow on the medium recommended for the plate count: thermophilic bacteria, for example, are best studied with a microscope. Again, obviously, in pasteurized milk only living bacteria can be detected by the plate count. Furthermore, no method

[2] American Public Health Association. Standard methods of milk analysis. American Public Health Association. New York, N. Y. 6th ed. 105 p. 1934. (7th edition in press.) To avoid repetition of the full title, the abbreviated term *Standard Methods* is used instead in some places in this circular.

tells much more than mere numbers. The presence of disease-producing bacteria cannot, as a rule, be determined. That is another story.

On the whole, however, these methods have made possible the production of clean, wholesome milk, and clean milk must be produced—milk as free from bacteria as human skill can make it.

The Nature of Bacteria.—Before one can easily comprehend *Standard Methods* or intelligently perform the various necessary tasks, he must have some concept of bacteria, which play a tremendous rôle in the dairy industry and in every walk of life—why, we shall presently see.

Bacteria are small—about 1/25,000 inch in length. The word *length* is used, but *diameter* might be substituted in some cases because bacteria, as found in milk, are both rod-shaped and spherical (fig. 17). The rods are called bacilli (singular, bacillus); the spheres, cocci (singular, coccus).

One important characteristic of the bacilli (but not of the cocci) is their ability to form spores. Spores develop by the concentration of the cell substance into hard, dense bodies having extraordinary resistance to usual lethal agents such as heat, drying, and germicides. One spore and only one is formed within a given cell. When conditions are again favorable for growth each spore can germinate and again becomes a rapidly growing organism. Generally speaking it is the spore which survives the "sterilization" process on the farm and in the city plant.

The smallness of bacteria, strangely enough, is a reason for their power. Thus a single microbe weighs 1/500,000,000 milligram. Its surface is, however, 1/100,000 square millimeter. This gives a ratio of surface to weight of 5,000 to 1. The same ratio of surface to weight in man is 0.025 to 1. Bacteria, then, have *relatively* 200,000 times more surface than man. This is more than mere arithmetic; it is a fact of great importance. Bacteria work through their surface. Food goes in through this surface. Roughly, the greater the surface, the greater the food intake. It has been proved that bacteria can digest *twice their weight* of lactose *per hour*. This they do hour after hour. No wonder the surface is important! Ferments, too, the agents of bacterial action, are produced in an abundance comparable with the consumption of food. These ferments (enzymes) pass out through this surface to ripen cheese, to sour milk, to flavor butter, and to do countless other things.

Even so, bacteria would not be highly important except that they multiply with amazing rapidity. One of them will split into two in 30 minutes (although it might be 15 or 60 minutes). At first this fact, too, seems meaningless—a mere statistic. Let us, however, assume a generation time (the period it takes one organism to become two) of 60 minutes.

Evidently in 48 hours (the usual period of incubation) one of these bacteria will divide 48 times. To reach some concept of the number of descendants a single cell will have in this short time, the reader may multiply as follows:

$1 \times 2 = 2$
$2 \times 2 = 4$
$4 \times 2 = 8$, etc., for 48 such multiplications.

The result staggers the imagination. If this process were to go on endlessly—or even for a week—there would be bacteria enough to fill the oceans of the earth. Nature, however, applies a brake: after this division has continued for a few hours, it stops. But already, as a rule, the damage has been done.

Bacteria are also important because they like the same foods as do human beings. In consuming this food they usually render it unfit for man. (Exceptions are sauerkraut, buttermilk, cheese, and the like.) There is hardly a human food that bacteria cannot also use.

In the laboratory where bacteria are studied they must be fed. Obviously one can feed them anything that man can eat. They would grow on a broth made from cabbage, from beets, from liver, veal, or pork. In the laboratory, however, we are taught to prepare their food from certain definite ingredients, and it is called a "medium." Directions are given which all must follow. Consequently, anyone making a medium anywhere, will have something as nearly standard as possible.

The standard medium contains peptone, meat extract, and agar dissolved in water. The amounts will be given later. Now all we need to know is that peptone is a protein, digested to make it soluble in water. Meat extract is just what the name implies: meat extracted in water. The agar is the dried juice of a seaweed with a jellifying property similar to that of gelatin.

The plate count utilizes this jellifying property of agar in the following way: Dilutions of the milk are made in water. Portions of this diluted milk are placed in petri dishes. Then into these dishes the agar medium is poured. It cools and sets. The bacteria in the sample are then trapped in the agar. They cannot move, but they feed and grow. After 48 hours a single cell, too small to be seen, has so many descendants that the *mass* of them is visible to the naked eye.

An analogy might be made at this point: To a military observer in a balloon 20,000 feet in the air, a single soldier on the plain below would not be visible. An army of 100,000 men marching there could, however, be seen. So it is with bacteria.

This point is made still clearer by figure 1,*A*. Here the cross section

of a plate is drawn. Four small dots in the agar represent four bacteria trapped or seeded there when the agar cooled. Even these small dots are, of course, much larger than the bacteria they represent. In figure 1,*B* the same plate is indicated after having been in the incubator for 48 hours. Now each organism has millions of descendents. This mass of bacteria, the army in the analogy above, is the "colony" one counts.

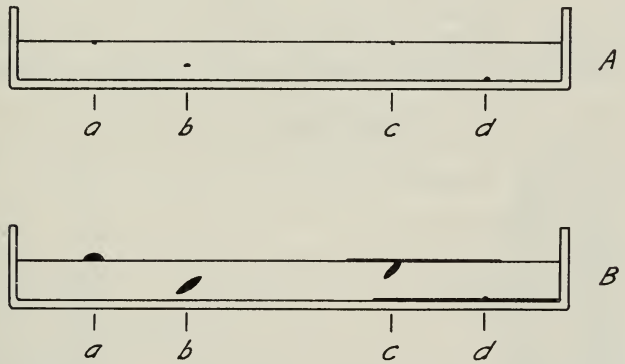


Fig. 1.—Cross section of a petri dish. In *A*, the dots above *a*, *b*, *c*, and *d* represent the position of bacteria as they might be trapped in the agar. In *B*, these bacteria have now grown to colony size by incubation and the colony can be seen for counting. There may be surface colonies (*a*) and subsurface colonies (*b* and *c*). The colony at *d*, in *B*, has grown between the agar and the glass and has a translucent appearance.

The rate at which this colony develops—that it, the number of times one organism divides to give the great numbers we see as a colony—is a function of temperature. With higher temperature (37° centigrade) there is a greater multiplication; with lower temperatures, less. To have the conditions of incubation the same in all laboratories a "standard" temperature (37° C) is designated. *Standard Methods* is exact on this point.

Thus ends our brief discussion of bacteria in the abstract—necessarily brief, since this circular is primarily an interpretation of a laboratory technique. We are now ready to look into the laboratory, to equip it, and to begin work.

THE LABORATORY

The Room.—A laboratory may be white-tiled throughout, equipped with polished metal autoclaves and stills; or it may be any room set apart from the rest of the plant for the sole purpose of controlling the quality of the incoming milk and the efficiency of the equipment used in the plant. The outlay for installation depends on the management's point of view. The laboratory may be a much-advertised display or an unassuming work room. The display idea is excellent, since it shows the pub-

lic that the distributor is attempting to protect the quality of his product and the well-being of his patrons. Such a laboratory is, however, expensive to install and maintain.

Whatever its cost, the room should be clean, well ventilated, light, and dry. Accessibility should be stressed. There should be freedom from drafts, which cause dust to rise to make trouble. A room 12 feet by 14 feet seems to be about the right size. Anything smaller would handicap the analyst and would make the heat from autoclaves and sterilizers unpleasant. The plan in figure 2 could be used. Attention should be given

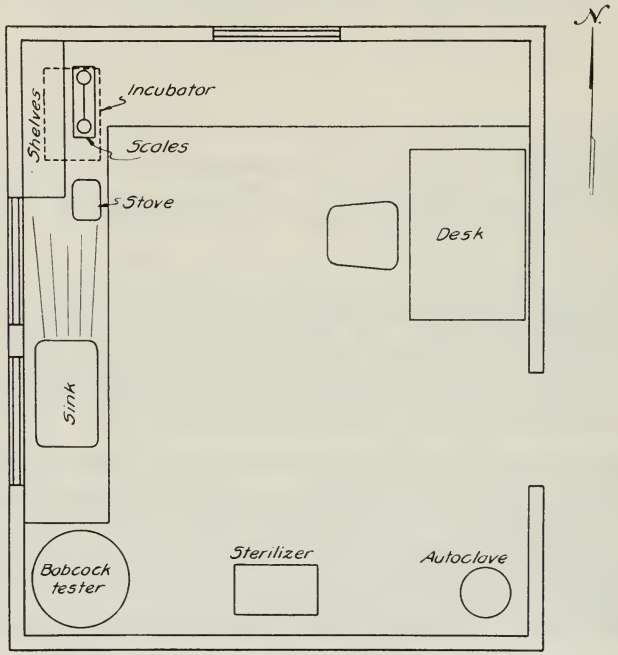


Fig. 2—Plan of laboratory for a plant where both bacteriological and fat tests are to be made.

to the flow of work through the room, and the apparatus arranged accordingly. Gas, electricity, water, and steam should be available. The table tops should be stained with aniline black. For this staining, two solutions are made:

Solution A	Aniline	120 grams
	Hydrochloric acid (commercial)	180 grams
	Water	1,000 cubic centimeters
Solution B	Sodium dichromate	120 grams
	Hydrochloric acid	100 grams
	Water	1,000 cubic centimeters

Solution A should be applied with a brush to the fresh, smooth surface and allowed to dry overnight. The color will turn bright yellow. Solution B should then be spread on the wood, which will turn dark and be very streaky at first. After this second coat dries, the surface should be rubbed with vaseline, motor oil, or paraffin. Vaseline seems preferable.

The Equipment.—The purchase and installation of the equipment should be carefully considered. Each article has its specific function, and this function should be understood. Styles, models, and types will be left to the individual. Catalogs should be consulted; salesmen may be asked to help. One may also visit a laboratory where work of this kind is done. A list of equipment with approximate cost is given below:

Article	Number[3]	Cost per unit
Autoclave	1	$35.00–500.00
Stove (2–3 burner)	1	6.00
Double boiler	1	2.00
Sterilizer (hot-air type)	1	45.00–500.00
Incubator, electric	1	80.00 up
Scales	1	9.00
Weights, 1 gram—100 grams	1	2.00
Funnels, 6 inch	2	0.30
Funnels, 8 inch	2	0.40
Funnels, 3 inch	6	0.10
Bottles (6-oz. prescription ovals)	1 gross	4.50
Bottles (2-oz. prescription ovals)	1 gross	3.50
Graduates, 1,000 cc	1	2.00
Graduates, 500 cc	2	0.75
Graduates, 100 cc	3	0.35
Flasks, 1,000 cc, Pyrex	24	0.50
Flasks, 200 cc	36	0.30
Petri dishes, 100 × 15 mm	*n* 15	0.18–0.30
Pipettes, 1 cc	*n* 15	0.18
Pipettes, 100 cc	2	0.25
Pipettes, 10 cc	10	0.20
Pipette box	2	3.00
Counting lens	1	3.00
Counting plate	1	0.15
Counting hand tally	1	5.00
Test tubes, 6 × ¾ inch	1 gross	2.80
Thermometer, 10° to 100° C	2	2.20
Thermometer, 0° to 250° C	1	1.50
Microscope	1	125.00
Breed pipette	1	1.25
Microscope slides	1 gross	$1.35

[3] "*n*" opposite "petri dishes" and "pipettes" refers to the number of samples to be plated daily. Six samples daily would require 6 × 15 = 90 plates to keep the work going.

To this list should be added as property constantly being replaced:

Article	Cost per unit
Difco peptone	$6.00 per lb.
Beef extract	$2.00 per oz.

Filter paper, cotton absorbent, cotton batting, pinchcocks, rubber tubing, rubber corks, cork stoppers, wax pencils, pH indicator brom thymol blue, dropping bottles, stains (ingredients for Newman stain).

The autoclave will be the biggest and most expensive piece of equipment. Prices range from $100 to $800. The autoclave is used for sterilizing fluids particularly. In it steam is confined so that a pressure is built up within the chamber resulting in a corresponding rise in temperature. Table 1 is given to show the relation of temperature to the pressure. In actual practice there is a lag in this temperature rise, however, especially if not all the air is excluded from the autoclave. More recently thermometers have been installed on these autoclaves.

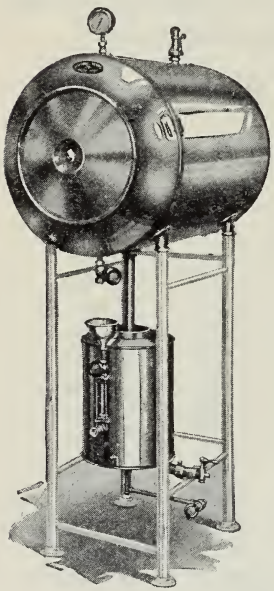


Fig. 3.—Autoclave such as is used in most bacteriological laboratories. This picture does not show the much-needed thermometer.

The theoretic temperatures for saturated steam and the steam pressure as indicated on the gauge will most nearly coincide with a heavily loaded autoclave after about 60 minutes of indicated pressure. The time depends largely upon the temperature, the mass and nature of the contents of the autoclave (thermal conduction rate), and the gas-supply rate—or on the pressure in the steam line when the latter is the source of heat. The time necessary for arriving at approximate coincidence is also influenced by the room temperature, the initial temperature of the autoclave and of the water in the generator, the weeping of the safety valve, and other factors.

Most autoclaves now have both temperature and pressure gauges. The following are suggested precautions to observe in the use of the autoclave:

When sterilizing *empty* plugged test tubes, flasks, and the like, invert them so that the stoppers are downwards, to insure more rapid and complete evacuation of contained air. Such glassware, when not inverted, should be loosely covered to prevent undesired water of condensation from dripping in.

Sterilization is accomplished more quickly and dependably when a large number of small filled flasks are used in preference to a small number of large flasks. To insure most rapid and thorough penetration of heat, large flasks should be only partly filled.

After the valve is wholly or partly closed, the steam pressure will rise rapidly and must be controlled so that it does not reach the blowoff point of the safety valve. The temperature will rise slowly until it reaches the optimum. Its rate of ascent will be influenced by the initial temperature and mass of the load and by the thoroughness of the air evacuation. Any

TABLE 1

Relation of Pressure to Temperature

Pounds pressure (above atmospheric pressure)	Degrees, Fahrenheit	Degrees, centigrade	Pounds pressure (above atmospheric pressure)	Degrees, Fahrenheit	Degrees, centigrade
0	212.0	100	11	241.6	116
1	215.3	102	12	243.7	117
2	218.5	103	13	245.8	118
3	221.5	105	14	247.8	120
4	224.4	107	15	249.7	121
5	227.2	108	16	251.6	122
6	229.8	110	17	253.5	123
7	232.4	111	18	255.3	124
8	234.8	112	19	257.1	125
9	237.1	114	20	258.8	126
10	239.4	115	..	.....	...

marked increase in mass and decrease in the initial temperature of the load from that of a known or control load will add considerably to the time necessary for completing the sterilization process.

After the optimum temperature has been maintained for the period of time essential for the thorough penetration of steam and the complete destruction of the organisms, cut off the source of heat and allow the steam pressure to recede. An alarm clock or "internal timer" is helpful in controlling the optimum period.

If the steam pressure is not carefully controlled as suggested and, as a result, the safety valve is allowed to lift, water will be primed into the sterilizing chamber through the steam shaft because of the sudden relief of steam pressure. Liquid culture media will also boil over, and sealed ampoules containing volatile liquids may explode. These accidents must be avoided.

Do not employ either the safety valve or the exhaust valve as a means of adjusting the optimum pressure since this is conducive to priming and wastes much steam. There is unnecessary wear on the safety-valve seat;

unnecessary strains are communicated to the sterilizing chamber; the contents of the autoclave will be saturated with water; and saline solutions or other corrosive liquids may be forcibly ejected from containers with possible resultant injury to the autoclave shell and valves. The ac-

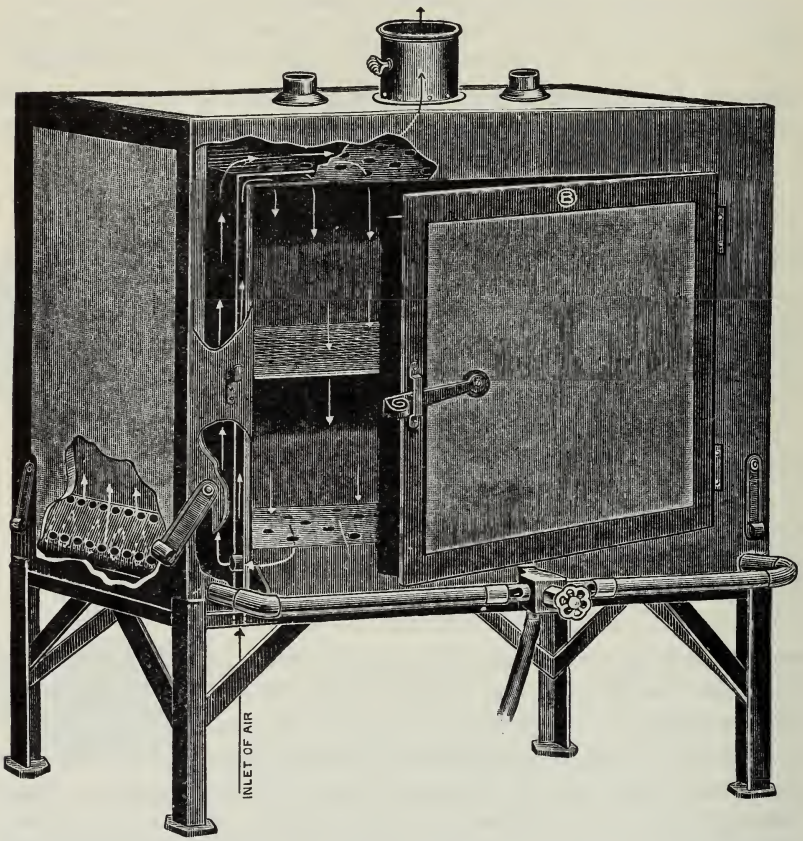


Fig. 4.—An improved type of hot-air sterilizer. It has a triple wall through which the heated air is circulated. The gas burners are not directly below the chamber, which is an advantage.

tual time of autoclaving of media as defined in *Standard Methods* is 15 pounds for 20 minutes after the pressure has reached 15 pounds.

Glassware (petri dishes, pipettes, etc.) is sterilized in the hot-air sterilizer. Regulation laboratory sterilizers (fig. 4) are comparatively expensive. As the cut shows, they have triple walls. Cheaper substitutes are on the market. In buying electrical sterilizers, one should note the position of the heater element. If it is directly below the bottom shelf, the equipment on this shelf will get more than enough heat; that on the upper

shelves, not enough. A sterilizer of more than ample capacity for the work should be bought in order to avoid crowding on the shelves. A temperature of 170° C for not less than one hour is the very minimum for sterility.

If any one piece of equipment is "most important," it is the incubator (fig. 5). In it a constant temperature is, or should be, maintained. All possible care should therefore be taken in its selection.

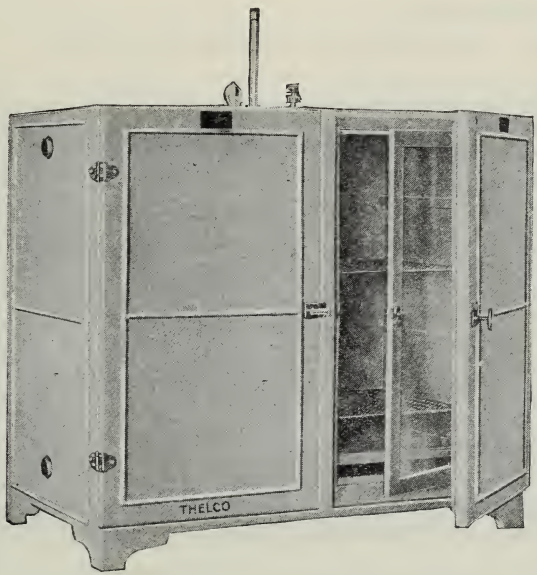


Fig. 5.—One type of incubator in use in bacteriological laboratories.

Judging from reports of the New York Agricultural Experiment Station, the ideal incubator is not to be found. Investigators in that institution studied the constancy and uniformity of temperatures in fourteen bacteriological incubators of different types of laboratory models partially or completely filled with duplicate agar plates prepared from samples of milk. Temperatures in all parts of the incubators were estimated from colony counts and from temperature readings. A perfectly uniform temperature of 37° C for 48 hours was never attained throughout the working-shelf space of the incubators studied. Temperatures were more uniform in incubators of the water-jacketed type than in those of the anhydric type. The petri dishes warmed more slowly in the the former type, because of the lower temperature of the heating surface. Least uniform temperatures were obtained in incubators with high-temperature heating surfaces and no provision for direct convection currents.

The proper ventilation of incubators is an important problem. Where a large number of agar plates are present, lack of ventilation may result in excessive humidity and may favor the development of spreader colonies, especially if the incubating chamber is overloaded. Too much ventilation may cause excessive drying of agar in the plates. If excessive amounts of unheated air are allowed to enter the chamber, the plates in immediate contact with the incoming air currents may be incubated at too low a temperature.

Apparently, exact control of temperatures is difficult to attain. The best one can do is to carefully select the incubator best suited to one's demands. Here, as with the autoclave, advice should be sought at laboratories where similar work is done and from trustworthy salesmen. In large laboratories whole rooms are sometimes given over to incubator use. Unless the need for space is great this arrangement is not advised, since temperature control in such a room is obviously more difficult than in small separate incubators.

Distilled water should be used for making media. Either a still should be installed, or some arrangement should be made for purchasing the water itself. In any case, the supply should be abundant and easily accessible.

Petri dishes (sometimes called petri plates or culture dishes) make a considerable item of expense because of the large numbers needed. About 20 must be kept in use per sample plated—not because so many are used at any one plating time, but because some will be in the incubator, some ready for counting, and still others in the process of washing and sterilization. This constant circulation in the laboratory leads to a high breakage unless the best materials are used. Heretofore these dishes have been made almost exclusively of a high-grade resistance glass. Recently, however, the makers of Pyrex glass have been able to pass along to the consumer certain manufacturing economies so that the cost of excellent dishes of this ware is now little more than that of ordinary glass.

Pipettes (1 ml), too, are constantly being washed and sterilized. They should therefore be purchased in numbers comparable with those suggested for the petri dishes. *Standard Methods* carefully defines the size and shape of these pipettes. Since manufacturers now conform to these specifications, reliable supply houses can furnish them with maximum errors of less than 0.05 milliliter.

Pipettes for sampling milk are of glass or aluminum with straight sides, long enough to reach the bottom of the original container. The size ordinarily convenient for 40-quart cans is 22 to 24 inches long with an inside diameter of ¼ inch. Aluminum tubes should have walls at least

$\frac{1}{32}$ inch thick, for they readily become soft and flexible at the higher temperatures of hot-air sterilizers. For this reason autoclave sterilization is recommended for metal tubes.

The equipment should include various sizes of jars and bottles. Screw-top jars are used for collecting samples. Since figure 6 suggests what they should be like, and *Standard Methods* describes them in detail, no more need be said here.

Prescription bottles (6 ounces, and 2 ounces) are used for various purposes in the laboratory—to hold media and dilution water. Some

Fig. 6.—Types of bottles and jars used for collection of sample (*F*) and for diluting the milk for plating (*A–E*). This shows, as well, the various closures used: *A* and *E* screw top; *B*, a wine cap of aluminum; *C* and *D*, aluminum foil. In *D*, the foil is raised to serve as a hood during sampling.

operators prefer round bottles, some oval. For the 2-ounce bottle the round form seems better, being less easily upset. In the process of plating, the caps of the dilution bottles are frequently removed and replaced. For that reason the method of closure becomes important. A cotton plug should never be used. Other possibilities are suggested in *Standard Methods*. Some use rubber stoppers. The crown seal, like that on a soda bottle, is also suggested. This means that a bottle opener is necessary, and the method of reseating the cap becomes a problem. Those who use the crown seal seem, however, to like it.

In California there has been developed a new type of closure—the wine cap (fig. 6), which is easily placed on the bottle and is as easily removed. The fact that it is not a tight seal does not seem to matter. It has been used for years with no reports of contamination. One drawback is its tendency to leak during the process of shaking. Its advocates contend,

however, that errors resulting from this leaking are more imaginary than real.

A modification of the wine cap is the use of aluminum foil, 0.00065 inch thick, which comes in rolls. The roll is attached to the laboratory wall, and portions are torn off as needed. One advantage is the hooded effect obtained during sampling.

In the author's laboratory the screw cap is used. Although its unscrewing would seem to slow the operation of plating, actually little time is lost. Further, the freedom from leakage is an advantage.

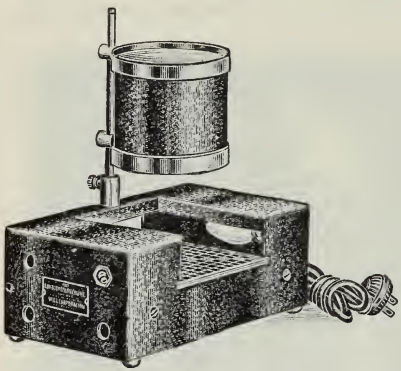


Fig. 7.—One of the many types of colony counters. It consists of a marked glass plate illuminated from above, or from either side. The petri dish is observed through the large lens held in a cylindrical device.

Since the plate count is really the enumeration of the colonies that develop on the agar in the petri dish, the apparatus used for this enumeration becomes of interest. Among the essentials of a good counting device are good lighting and a dark background. Some operators prefer to have the light come from above; some, from below. Apparently the preference is a matter of experience and training. In any case artificial illumination of the plate is essential. According to some workers, light from above does not illuminate pinpoint colonies successfully.

Many colony-counting devices are on the market (fig. 7)—some elaborate and expensive. No recommendation will be made here. An inexpensive and convenient device is illustrated in figure 8; it may be described as a framed counting plate sliding in guides below a shelf, but above a lamp. The light illuminates the plate from below. When the lens is used the black table top is thrown out of focus, which gives an ideal background. When not in use, the plate can be slid back under the shelf out of the way. This same light, incidentally, could be used for the microscope work, to be described later. The plate marked in squares (Wolfhügle) seems preferable to the one marked in circles (Jeffers). With the square plate one starts at one point on the petri dish and ends at another. With the Jeffers plate one starts and finishes at the same place and, frequently, tends to count beyond the starting point.

A 4-inch reading glass (magnification of 1½ diameters) is used to observe the colonies. The tallying is made with a Veeder hand counter. A counter with a "quick set back" is perhaps preferable.

Thus ends our list of essential supplies for a bacteriological laboratory.

Preparation for Plating.—The laboratory is now ready for occupancy. Before carrying out the actual plating operation we must make certain preparations: The glassware (such as pipettes and petri dishes) must be washed and dried. They must be placed in containers and sterilized (at not less than 170° C for not less than one hour) in the hot-air oven.

Various sizes of these containers are to be found in dealers' catalogs. Three-pound coffee cans make splendid containers for the dishes. In

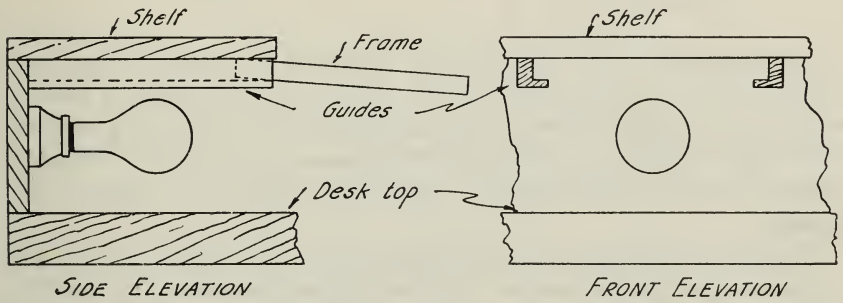


Fig. 8.—A conveniently arranged colony-counting apparatus. It is simple and inexpensive.

special instances petri dishes can be left free on the shelves of the sterilizer; this procedure is not to be recommended as a general custom unless very little time elapses between sterilization and use.

Sample bottles must be sterilized. If they have paper or rubber gaskets, the autoclave should be used. Sampling pipettes should be wrapped in paper or placed in large containers.

The real preparation now comes in filling and sterilizing the dilution bottles and in making and sterilizing the media.

In filling the dilution bottles, distilled water should be used. Because of the slight loss in volume during autoclaving, the exact amount of water to be added to the bottle before sterilization should be determined experimentally for the particular autoclave in use. These dilution bottles should have 99 milliliters of water *after* sterilization (9 milliliters for the 2-ounce size).

As stated in *Standard Methods* all dilution bottles should have 99 milliliters or other graduation marks placed on them, and observations should be made before use on each bottle in order to determine that it actually contains 99 milliliters of sterile water. A tolerance of $\pm$ 2 milliliters is allowed.

These bottles then should be capped according to the discussion above and placed in the autoclave for sterilization.

The medium must now be prepared. It is the crux of the whole plating method; upon its constancy of ingredients and reaction much depends. Strict adherence to the formula given below is necessary for satisfactory results.

The medium has the following composition:

Peptone	5 grams
Meat extract	3 grams
Agar	15 grams
Water	1,000 milliliters
Reaction	pH 6.8

The ingredients have already been discussed in a general way, but definite information will now be given. Although many different brands of peptone are to be found on the market, *Standard Methods* decrees that "Bacto"[4] peptone or "any other peptone giving equivalent results" should be used. Unfortunately, "equivalent results" are hard to determine.

The agar is procurable both as threads and as fine flakes. The longer threads are preferable because they lend themselves better to a washing process necessary for certain special media.

The reaction (pH 6.8) is a definite requirement. It determines the degree of acidity in the medium. Fortunately, the peptones of today are made so that their solutions are of the reaction desired. A medium prepared with them rarely needs adjustment. The reaction should be checked, however; and unless one is familiar with this procedure some difficulties might arise.

Hydrogen ion reactions, or the "pH" as it is sometimes called are easy to understand. Without going into the basic theories of the reactions involved, one may say that the idea is to determine the weight of hydrogen ions in a liter of water or solution. The thermometer, as is well known, is graduated in parts known as degrees. In a similar way the acid-alkali scale is graduated, so to speak, in "degrees" called pH values. There are fourteen of these, from pH 0, a solution of approximately normal acid (36.5 grams of concentrated HCl in 1,000 cubic centimeters water), through pH 7 (the "neutral" point), to pH 14 or approximately normal alkali (40 grams sodium hydroxide in 1,000 cubic centimeters water).

In determining the pH of a solution several indicators are used. Each

[4] Bacto peptone is made by the Difco Laboratories of Detroit, Michigan. Sometimes the word "Difco" peptone is used, and misunderstanding may result. "Difco," a registered trademark, is not correctly applied to any product except as it follows its name—thus, Bacto Peptone Difco. Other peptones manufactured by this company need not be confused with Bacto, since they have easily recognized names of their own.

of them displays two colors—one an acid color, the other basic. There is a definite place in the pH scale (indicated by rectangles in figure 9) where for each indicator the transition from one color to the other takes place.

Although many indicators are known only those have been selected which have general acceptance.

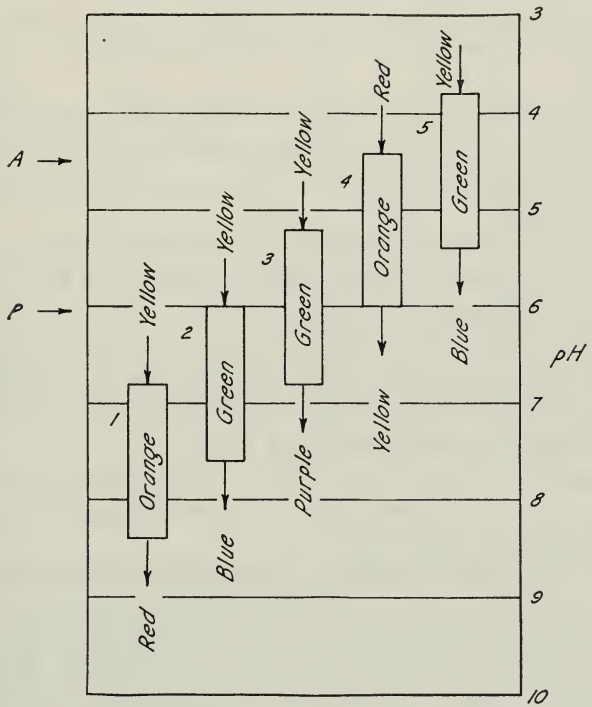


Fig. 9.—Zones through which each indicator changes its color from yellow to red, or yellow to blue as the case may be. See text for explanation.

In figure 9 these indicators are represented as vertical areas, numbered from 1 to 5. These numbers correspond to the list of indicators given below. The acid color for each indicator is given above the numbered area and the basic color below.

The ends of these areas are drawn at the place on the scale (in figure 9) where the full acid (or full basic) color only is seen. Within these areas (from top to bottom) the colors change from yellow through orange to red or undergo whatever other change is indicated. As will be noted, these zones overlap.

The list of indicators, together with the pH range is here given:

1. Phenol red—pH range of 6.8–8.4.
2. Brom thymol blue—pH range of 6.0–7.6.
3. Brom cresol purple—pH range of 5.2–6.8.
4. Methyl red—pH range of 4.4–6.0.
5. Brom cresol green—pH range of 3.8–5.4.

If a solution of pH 4.5 (*A*, on the left-hand scale in figure 9) were taken as an example, indicators 1, 2, and 3 would be yellow (full acid color); 4 would be red (acid color); and 5 would be green (a transition color).

If now, we added small amounts of a weak alkali to this solution, the pH would slowly change. This change would be noted in indicator 4, which would turn progressively more orange; and in indicator 5, which would become bluer.

At pH 6.0 (*B*, in figure 9) indicators 1, 2, and 4 would be yellow; 5 would be blue. Indicator 3 would tell the story of the pH. At pH 6.8, indicator 3 would be green. It is the one to use.

A thorough study of this chart is suggested. In brief, each indicator has telltale colors indicating the pH of a solution. In actual practice, where media making alone is under consideration, the process is easy. Only one indicator (brom thymol blue) is used.

These slight variations of color are not easily kept in mind. To aid the worker, an artifice is employed that closely accords with absolute values known to more exact work.

A series of 6 test tubes is placed in a rack, and to these is added a weak solution of hydrochloric acid (1 drop of acid to 100 milliliters of water), 5 milliliters to a tube. In another set of 6 tubes in front of these is placed 5 milliliters of 0.05 per cent sodium hydroxide solution. To the first tube of the acid series is added 9 drops of the indicator brom thymol blue, to the next 8 drops, then 7 drops, and so forth. The intensity of the yellow color decreases as less and less indicator is used. Now, to the series of alkali tubes, 1 drop of indicator is added to the first tube, 2 to the second, and so on; the intensity of blue increases as more of the indicator is added.

When the work is finished there will be 6 pairs of tubes, and the numbers of each pair will have 10 drops of indicator between them (9 in the acid tube plus 1 in the alkali tube, or 8 plus 2, and the like). When one looks through any pair of tubes the shades of green will correspond to certain pH values.

Figure 10 brings this situation out more clearly. The pair of tubes marked *A* (reading down) having between them 10 drops of indicator

will correspond in color to pH 6.2 when they are observed one behind the other. If the color of the indicator in the medium does not correspond to the color of the tubes between pH 6.6 and pH 7.0, adjustment is necessary. To adjust the medium, $N/20$ NaOH[5] is added from a burette

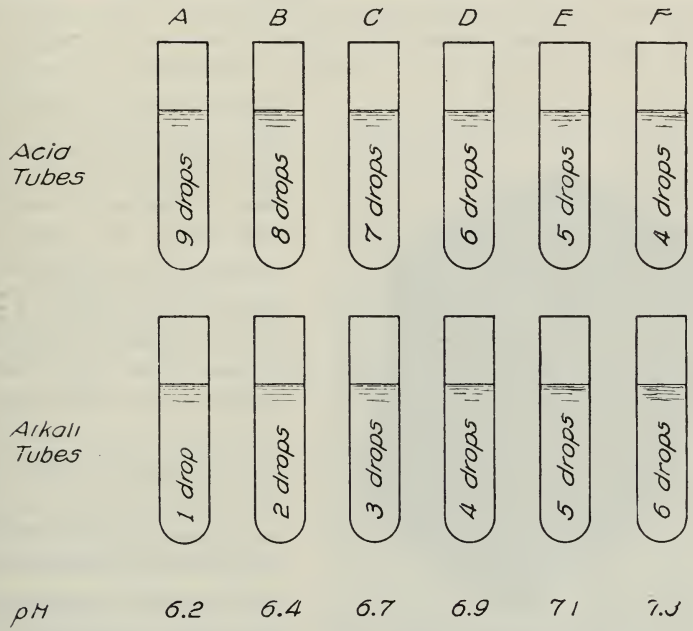


Fig. 10.—The drop ratio method of determining pH values. See text for explanation.

or pipette to the tube until the desired shade of green is obtained. Note the amount of $N/20$ NaOH used. Then add 50 times this amount of normal NaOH ($N/1$) to a liter of the medium, which will then have the desired pH. This determination should be made after as well as before sterilization.

To perform this operation better, a comparator block is used (fig. 11).

Figure 12 indicates the top of this comparator block. The various holes are numbered. In 4 and 7 are placed an acid and alkali tube from the drop-ratio series (say the pair designated *C* in figure 10). In holes 6 and 9 the pair designated *D* are placed. In holes 1 and 3, tubes of medium are placed because the color of the medium must be considered. In hole 5 the medium to be tested is placed. It, of course, has 10 drops of the indicator. Tubes of water are now placed in 2 and 8.

[5] $N/20$ NaOH is made by dissolving 2 grams of sodium hydroxide in 1,000 milliliters of water. $N/1$ NaOH is 20 times stronger. For such work as that described above, these solutions need not be corrected, though correction would be necessary if refined work were done.

When once the theory of indicators is understood, the making of the medium is the next concern.

A suitable pair of scales should be available. The ingredients are weighed with care. Correct amounts are added to the distilled water. The mixture is heated until the agar is "dissolved." Too much boiling should be avoided lest valuable nutritive substances be coagulated or lost. After the agar is dissolved, acidity is checked. If the reaction is between pH 6.4 and pH 7.0, there is no need for adjustment. The medium is now clarified or filtered and dispensed into bottles, care being taken to put not more than 180 milliliters into any bottle. The bottles are plugged with cotton or capped; wine caps, however, should not be used here. The bottles are now placed in the autoclave and sterilized at once before the agar has a chance to cool and jell. When jelled it is much more difficult to sterilize since heat penetrates fluid agar more readily than solid.

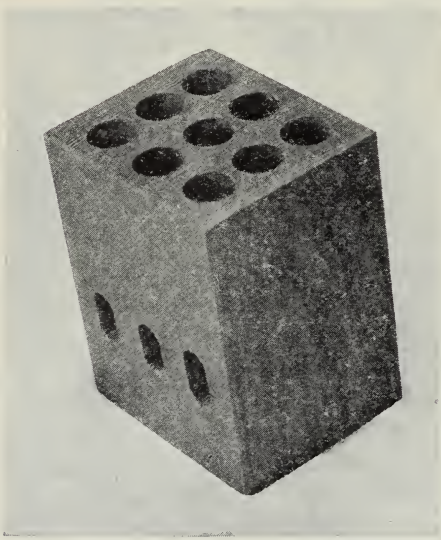


Fig. 11.—The comparator block.

After sterilization, the agar should be removed from the autoclave and allowed to cool. The melted agar should not remain in the autoclave in the melted condition, lest the jellifying properties be injured. It is wise (though not necessary) to store the agar in an ice box. In any event, the agar must not dry out.

One can avoid the rather arduous task of medium manufacture by using dehydrated media, also a product of the Difco Laboratories. It is an agar medium, standard in every way, made in large quantities by controlled methods and then dried to a powder. Its use is permitted by *Standard Methods* and is elsewhere highly recommended. It is somewhat more costly than a medium made from the ingredients in the laboratory. If, however, time is at a premium, the added cost is justified.

At last the laboratory is finished, the medium is prepared, and everything is ready.

OPERATION OF THE LABORATORY

Collecting the Sample.—One must first get the sample of milk to plate. Obviously, one secret of accurate counting is to choose a sample truly

representative of the whole. Evidently, too, the slender sampling pipette is hardly large enough to stir a large can adequately. If a conventional metal can stirrer is used, the procedure according to *Standard Methods* will be as follows:

Satisfactory facilities for stirring the milk in the can may be made available by providing one can of clean, cold water through which cold water passes continuously and one can of hot water kept at a temperature of at least 180° F by passing live steam through it. The milk is rinsed off the metal can stirrer in the can of cold water, and the clean stirrer is then allowed to stand in the can of hot water while the sample collector is preparing to take the next sample.

Samples from containers shall be removed with sterile glass or metal tubes, long enough to reach the bottom of the original container and inserted, not too rapidly, with the top of the tube left open. The finger is then placed on the top of the tube, making it possible to withdraw the tube full of milk and to transfer the entire contents of the tube to the sample vial or bottle.

Each sample shall consist of at least 10 ml of milk or cream. Vials and sample bottles shall not be filled more than two-thirds full, thereby permitting proper agitation when the samples are plated.

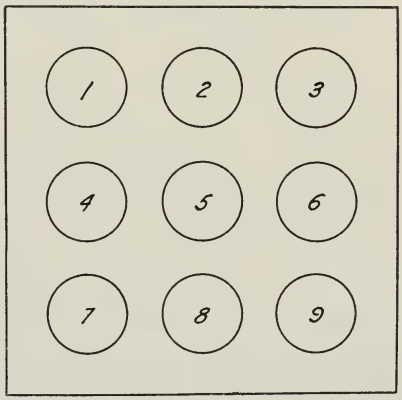


Fig. 12.—Designating the various holes in the top of the comparator illustrated in figure 11. Tubes are placed in these holes as described in the text.

Each sample bottle should be properly identified by a label. To prevent growth, these samples should be placed in ice as soon as taken. Cases for transportation of refrigerated samples will be left to the ingenuity of the individual.

Plating.—In the plating operation, order and system are essential. The table top is wiped with a damp (not wet) cloth to remove dust. Then the articles to be used are assembled there. An arrangement is suggested in figure 13.

A represents the petri dishes laid out. There are two dilutions, both in duplicate. Behind this is the sample to be plated (*B*). Behind this again are the dilutions (*C*). The pipette case is represented as at *D*; it projects slightly over the edge of the table, with about 1 inch of the pipettes exposed. The agar has been melted and placed in a water bath (*E*) at 40° C, ready for pouring. A wax marking pencil is shown at *G*. Sometimes a tray *F* is used for dirty pipettes. This arrangement can, of course, be duplicated as many times as needed.

In the plating operation bacteria are seeded in a nutrient agar, where

colonies develop. This process we have already described. Theoretically, one colony will develop from each bacterium (seed). If milk containing 500,000 bacteria per milliliter were placed in a petri dish, obviously such a large number of colonies would be impossible to count. For this reason a system of progressive decimal dilutions is used. If 1 milliliter of such milk (500,000 per milliliter) were added to 99 milliliters of sterile distilled water, it would make a 1 to 100 dilution of the milk *and the bacteria*. Each milliliter of the mixture would now contain 5,000 bacteria

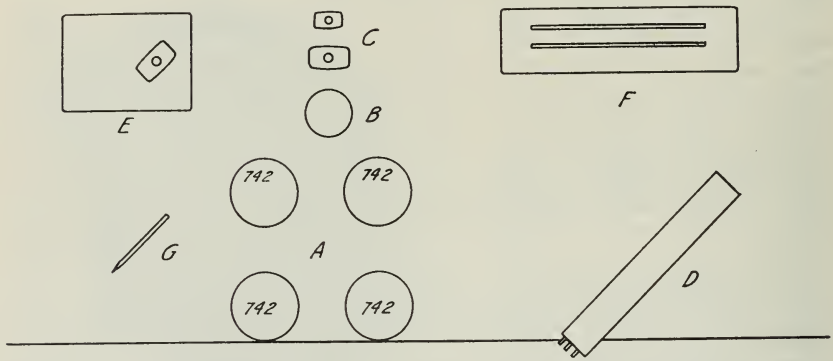


Fig. 13.—Arrangement of glassware and bottles for plating one sample of milk. The units in this arrangement can be duplicated as many times as desired. See text for explanation.

(1/100 of 500,000=5,000). If this were diluted again with 1 milliliter added to 9 milliliters of water, the original milk *and the bacteria* would now be diluted 1,000 times (1/10 of 1/1,000 of 500,000=1/1,000 of 500,000=500). Here, again, a little study might be necessary. Figure 14 makes clear this dilution technique.

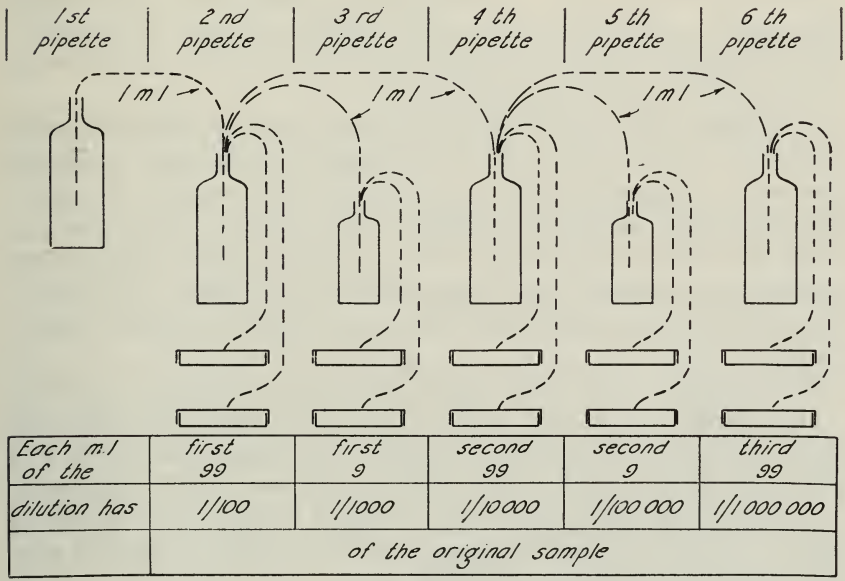
The analyst must decide upon the actual dilution to be used for a given sample of milk. Experience will show that while one patron, always a producer of high-count milk, will need dilutions of 1-10,000 or 1-1,000,000 to give a satisfactory plate, his neighbor never needs a dilution higher than 1-100.

The plates must of course be marked for later identification. Such marking must include the dilutions used. If one were to write the fraction (1-1,000, 1-1,000,000) confusion might arise. In writing such numbers and in reading them later, one could accidentally add or drop a cipher. Instead of 1-100, the letter "H" can be substituted to represent "hundred," or "C," for the Latin *centum* (hundred).

Still another way to designate these bothersome strings of zeros is to indicate their number (2 for 100, and so forth). The figures (2, 3, etc.)

can also be considered as the exponents of 10, which would give that number: thus, $100 = 10^2$. Tabulated below are the designations for various dilutions in all three systems, some one of which should be used:

Dilution	Initial	Latin	Exponent
1-100	H(undred)	C(entum)	2
1-1,000	T(housand)	M(ille)	3
1-10,000	OT	XM	4
1-100,000	OOT	CM	5
1-1,000,000	M(illion)	MM	6



Each ml of the	first 99	first 9	second 99	second 9	third 99
dilution has	1/100	1/1000	1/10000	1/100 000	1/1 000 000
	of the original sample				

Fig. 14.—If the sample to be plated is found in the first bottle to the left, the first pipette would place 1 milliliter of milk in the dilution bottle; or (reading in the box below the figure) "Each ml of the first 99 dilution has $\frac{1}{100}$ of the original sample." By referring both to the drawing and the box below it the idea of the dilution will be easily understood.

When the plates are marked and everything is ready, proceed according to the following directions, as given in *Standard Methods:*

When the milk is in sample vials or bottles, or in dilution bottles, each vial or bottle shall be rapidly shaken 25 times, each shake being an up-and-down excursion of about 1 foot (entire shaking not to take longer than about 7 seconds).

When measuring milk, the practice shall be to *blow* the last drop from the pipette and then to place the pipette in water immediately to make subsequent cleaning easier. After placing the milk in the dilution water, the practice shall be to allow the contents of the pipette to *run* into additional dilution bottles or into the petri plate, the end of the pipette touching the dish as the liquid runs out. Sufficient time shall be allowed to permit the draining of a complete ml of liquid into each dish. Use

care to raise covers of petri dishes only as far as necessary to insert the end of the pipette. Touching the tip of the pipette to a dry place in the petri dish offers greater assurance of complete delivery.

Never use a pipette in more than one dilution. If dilutions are transferred to the next bottle *and* to 4 to 6 petri dishes, care should be taken that the pipette touches nothing but the dilution water and the inside of the dishes.

Add the agar to the plates after the dilutions are in. Too much agar in the petri dishes leads to the development of "spreaders," the trial and worry of a careless operator. Spreaders, as one might guess, are colonies that spread over the surface of a plate, making an opaque mat that prevents counting of the other colonies. Spreaders can be caused also by unsterile utensils, dilutions, and the like.

Incubation.—As soon as the agar is cooled and solidified, the petri dishes are inverted and placed in the incubator, still inverted. Again the prevention of spreaders is the reason for inverting the plates. If a plate were placed agar-side down, the drops of condensation water collecting on the lid of the petri dish might fall on the agar surface. This accident could cause a spreader if the drops should strike a colony. If, however, the petri dish is inverted, such water as does collect remains where it formed.

Overcrowding of incubators must again be mentioned. Apparently it often leads to erroneous results, since, with overcrowding, the heat cannot penetrate well all parts of the space. As a result, some plates will receive less heat than others and will have correspondingly less growth.

As soon as the plates are in the incubator the laboratory should be placed in order. All used glassware should be cleaned in alkaline solution. It may well be rinsed in distilled water.

Since plates can rarely be counted exactly at the end of a 48-hour incubation period, a tolerance of plus or minus 3 hours of incubation at 37° C is permitted.

Counting the Colonies.—After the incubation period (48 hours), the plates are placed on the laboratory table. The counting apparatus is assembled. In the plates will be found colonies of bacteria varying in size from nearly invisible pinpoints to the diameter of wheat grains or larger. As will be recalled, these colonies are masses or communities of bacteria, theoretically, at least, the progeny of one organism. With the aid of the lens these are counted after the plate has been placed in the counting apparatus. Some operators simply observe one colony after another, pressing the tally counter as the eye picks up each colony. Some advise inverting of the plate and touching the glass with a pen as a check on what colonies have been counted. This method is somewhat slower,

and the extra time is probably not well spent. Frequently colonies at the very edge of the plate are missed. Still another method is to crush the colony with a sharp-pointed instrument as it is counted. This too is slow. With practice the operator can easily learn to check himself.

This operation in the plate count is somewhat difficult for the beginner. Sometimes he finds it hard to tell whether the object observed in the agar is a colony or a bit of debris in the medium. Despite precautions, debris is often present. Here, again, "practice makes perfect" or as nearly perfect as can be expected. Some one should, however, coach the uninitiated in the counting of colonies.

Some plates may be crowded (400—500 or more), while others may have very few colonies. *Standard Methods,* therefore, decrees that in preparing plates such amounts of milk should be used as will give not less than 30 colonies and not more than 300. This ruling, though arbitrary, should be strictly adhered to.

After the actual count is made (for example, 45 colonies) the number is multiplied by the dilution (say 1-1,000). The result is the reportable number (in this case 45,000).

Accurately speaking, the counts from agar plates give the estimated number of colonies that would have developed on standard agar per milliliter of milk if an entire milliliter of milk had been incubated under favorable conditions. Therefore, all agar plate counts obtained by the standard technique should be reported in the form "standard plate count, 20,000 per ml" rather than as "20,000 bacteria per ml." This expression "standard plate count" should be considered as an abbreviated method of saying "an estimated count of 20,000 colonies per milliliter as obtained by standard methods." Furthermore, the standard plate count should be "rounded out" to two significant figures. In the following numbers, 136, 107, 291, the 13, 10, and 29 are the significant figures; and the 6, 7, and 1 are assumed not to be significant within the accuracy of the method. In reporting such counts use 140, 110, and 290 (times the dilution); the count is to be lowered with numbers from 1 to 4 and raised with numbers from 5 to 9.

Never count two dilutions; always select one (with colonies between 30 and 300) and discard the other.

Quoting from *Standard Methods:*

> Agar plate "counts" per ml are to be regarded as "estimates" of numbers rather than as exact counts, since only a portion of a ml is used in preparing the plates. As such they are (like all estimates) subject to certain well known and recognized errors whose size can be controlled largely by the care taken in the analysis. Among these errors are: (a) failure of some of the bacteria to grow because the incubation temperature, or the composition or reaction of the medium is not suitable; (b) inac-

curacies in measurement of the quantities used; (c) mistakes in counting, recording data, computing results and the like; (d) incomplete sterilization or contamination of the plates, dilution waters, etc. The possible errors caused by these things make it highly important for all routine laboratories to follow the standard procedure carefully.

When the plate to be counted has developed more than 250 colonies it is a common practice to count a fraction of the plate and then estimate the total number of colonies on the plate. This procedure, too, is subject to error because of variations in the inside diameter of the bottoms of the petri dishes and also because workers are frequently careless in determining the factor that should be used in multiplying the average number of colonies per sq. cm. Because 9.1 cm has been found by actual measurement to be the average inside diameter of the ordinary type petri dishes, the average number of colonies per sq. cm should normally be multiplied by 65.0. If, however, the average inside diameter is only 9.0 cm, the factor should be 63.5; if 9.2, it should be 66.5.

The matter of records themselves—the forms used—is left to the operator.

THE DIRECT MICROSCOPIC COUNT

There are three criticisms of the plate count method: (1) Not all the bacteria are able to grow on the medium used. (2) Those that do grow take 48 hours to form a recognizable colony. (3) These colonies are not always the descendants of a single cell.

Obviously, if a method could be worked out whereby the individual bacteria could be rapidly stained and then observed directly with the microscope, then the faulty plate-count method could be abandoned. Dr. R. S. Breed and Dr. T. D. Brew of the Geneva, New York, Experiment Station, worked out such a method, variously called the Breed count, the direct count, and the miscroscopic count. It was fostered by Breed throughout many years of indifference of public health officials. Despite its merits, it has never supplanted the older plate count upon which so much dependence is placed. This microscopic count is merely the staining and observation of bacteria in a film of milk. Any one can make such a film or "smear." The service that Breed and Brew performed was to correlate the numbers of bacteria seen in such a film with the numbers found in a milliliter of milk. To make this correlation, a definite quantity of milk (0.01 milliliter) is spread over a definite area (1 square centimeter) of a glass slide. The resultant film, approximately 0.1 millimeter in thickness, is examined (after staining) with a microscope having a field of view of a definite size (0.205 millimeter diameter). The milk film thus observed through the microscope is 1/300,000 of a cubic centimeter. From the number of bacteria seen in this amount of milk one can easily calculate the number in the whole cubic centimeter.

This procedure is illustrated in figure 15. At the top is shown a microscope slide with the film of milk spread over 1 centimeter square. Below is a cross section of the slide along the line *A-B* with the milk film represented. The microscope objective is just above the film over the shaded portion. This shaded portion is next shown at the bottom (greatly en-

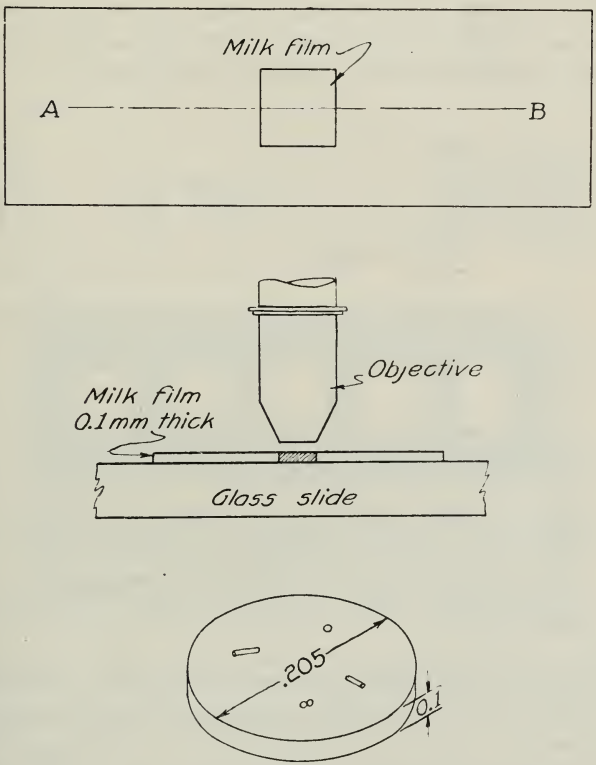


Fig. 15.—Top: Slide with a milk film spread over 1 square centimeter. Middle: Side view showing the milk film magnified by the objective. The shaded area is the portion of the milk film which is seen at any one time. Bottom: Greatly magnified representation of that portion of the film which is seen at any one time; it measures $\frac{1}{300,000}$ of a cubic centimeter.

larged) as a little pellet or button of milk 0.205 millimeter in diameter and 0.1 millimeter thick. This button is 1/300,000 of a cubic centimeter. From the number of organisms seen in this button (5 in this case) the number per cubic centimeter can be calculated by simple multiplication.

To standardize the microscope to a field size of 0.205 millimeter a stage micrometer must be used—that is, a microscope slide to which is

cemented a glass disk, ruled in tenths of millimeters and in hundredths (fig. 16). This slide is placed on the stage of the microscope, and the lines are brought into focus. The selection of eyepiece, objective and drawtube is made that will just fill the field with two large spaces and half a small one.

This condition is usually fulfilled when a 5× ocular and an oil-immersion objective are used at a drawtube length of 175 millimeters. As such an adjustment is difficult to describe, the purchaser should ask to have the microscope adjusted before it leaves the manufacturer.

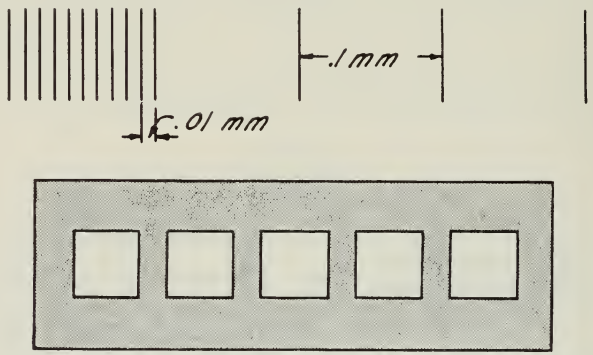


Fig. 16.—Upper: Markings to be observed on the stage micrometer. Two large spaces and one-half of the first small one will give the correct field size. Lower: A microscope slide etched about the edges but leaving clear windows of exactly 1 square centimeter area upon which the milk is spread.

Pipettes are now sold which are made to deliver 0.01 milliliter of milk. One should choose carefully, selecting only those with a ground-off, cone-shaped tip. The drop of milk should be discharged cleanly and should not run back on the side of the tip. As these pipettes vary somewhat, it is well to calibrate them by determining the weight of milk they contain. Each should deliver 0.0103 gram.

Only one pipette is needed for the work. Although it need not be sterile, it should be flushed out after each sample of milk by rinsing inside and out with warm, clear water. When not in use it should be kept in a glass-cleaning solution or in sulfuric acid.

The slides may be the common 1 × 3 inch microscope slides. If these are used a ruled card can be purchased to serve as a guide in the spreading of the milk. Slides somewhat larger but with the surface ground except for little windows of the proper size (fig. 16) are, however, decidedly to be recommended.

Before performing the microscopic count, prepare the Newman-Lampert stain as follows:

Methylene blue,[6] powdered	1.12	grams
Ethyl alcohol, 95 per cent	54	milliliters
Tetrachlorethane (technical)	40	milliliters
Acetic acid, glacial	6	milliliters

Add the alcohol to the tetrachlorethane in a flask and bring to a temperature not exceeding 70° C (with methyl alcohol the temperature should not be raised to more than 55° or 60° C). Add the warm mixture to the powdered methylene blue. Shake vigorously until the dye is completely dissolved; then slowly add the glacial acetic acid to the cold dye-solvent solution, keeping the latter agitated during the addition of acid. Filter the entire volume through a 15-centimeter filter paper. Keep in a tightly stoppered bottle. This stain fixes the film on the slide, removes the fat, and also stains the milk bacteria.

The steps in the microscopic method are as follows:

1. The milk is drawn into the pipette, and the tip of the pipette is wiped with a clean cloth.
2. The milk is blown onto a glass slide.
3. It is spread over an area of 1 square centimeter by means of a clean wire or needle.
4. It is dried on a level surface, care being taken not to dry it too quickly because such drying causes cracking.
5. The slide is placed in a Coplin jar containing the stain.
6. The slide is removed from the jar at once and allowed to dry. Drying is important because the solvent used in making the stain does not mix with water. If the undried film is placed in water, it either floats to the surface or is filled with grainy specks.
7. Wash in water.
8. Dry in air.

Figure 17 represents a microscopic field showing various types of bacteria. This drawing is made solely to acquaint the operator with grosser aspects of the different objects seen in the various fields of the microscope. *No one should attempt the microscopic count under any circumstances without working at least one or two days with someone who knows how.*

The bacteria in each of 30 fields are usually counted, and an average is taken. This average, multiplied by 300,000 gives the count per milliliter.

[6] Certified.

The method will not permit too close separation of grades of milk. One could not, in other words, distinguish between a milk with 18,000 bacteria per milliliter and one with 22,000.

Fig. 17.—Representing the gross appearance of objects seen in milk films at approximately 1,000 diameters: *a*, streptococci (chains); *b*, probably *Streptococcus lactis*, the milk-souring organism of the starter; *c*, short rods; *d*, leucocyte; *e*, clump of bacteria possibly dead but carried over into the fresh milk in the cans.

THE PROPER USE OF THE MICROSCOPE

When a microscopic count is made, one observes objects or images of objects which are 1 to 2 micra[7] in diameter or length. Microscopes normally used for such work are able to resolve objects of about a half micron. It is apparent that in counting bacteria in a milk film the microscope is being used practically at the limit of its power of resolution. Since one might easily mistake two adjacent cocci for a single bacillus, critical use of the instrument is required to avoid serious error.

The microscope is an instrument of precision and should be used as such. To operate a microscope properly one must follow a few simple rules based upon known optical facts.

Most microscopes used for counting bacteria in milk films have three objectives. Each objective has certain characteristics including the "working distance" and the numerical aperture (N.A.). Table 2 indi-

[7] 1 micron = 0.001 millimeter or 0.00004 inch.

cates the numerical aperture values and the working distance of the three commonly used objectives mentioned above.

Of the characteristics designated in this table, the N.A., is of the greatest significance, for it is a measure of the ability of a lens to resolve an image and hence a measure of the optical capacity of that lens.

Before a clear idea can be had of what resolving power means it will be necessary to understand three simple concepts. First it must be clearly understood that the refractive index of the medium through which light

TABLE 2

Working Distances and Numerical Apertures of Lenses of Varying Magnifying Power

Lens	Working distance, millimeters	Numerical aperture
Low power	16.0	0.25
High, dry	4.0	0.85
Oil-immersion	1.9	1.25

passes is of great importance to microscopy. The symbol n for this refractive index is found in many equations. Second, it must also be borne in mind that the wave length of light also plays a major rôle in optics. This wave length is designated as W in the equations that follow. For those unfamiliar with these terms and values two tabulations are appended, one giving values of n for various substances and the other giving values of W for the different parts of the visible spectrum. The refractive indexes for various substances are:

	Refractive index (n)
Air	1.00
Water	1.33
Glycerin	1.46
Cedar-wood oil	1.51±
Balsam	1.53±

The wave lengths for various forms of radiation are as follows:

	Wave lengths (W) in micra
X-rays	0.00001–0.015
Ultraviolet	0.29 –0.39
Blue	0.42 –0.49
Green	0.49 –0.53
Yellow	0.53 –0.59
Red	0.65 –0.80

Third, a statement is necessary defining the angular aperture (A.A.) of a lens as that angle subtended between the most divergent rays which

can pass through it. In the equations given below the term α (alpha) is

$$\text{half of the angular aperture or } \frac{\text{A.A.}}{2}$$

Two basic equations are necessary for explaining the relation of optics to correct illumination. The one for numerical aperture is as follows:

$$\text{N.A.} = n \sin \alpha \qquad 1$$

To those for whom mathematical formulas are anathema let it be said here that equation 1 simply states that N.A. increases with an increase in refractive index of the substance traversed. This equation explains why oil is used for the oil-immersion lens. If one were to substitute in this equation the values of N.A. (1.25 for the immersion lens) and 1.50 for the n (table 2) one would find that

$$1.25 = 1.50 \sin \alpha$$
$$\sin \alpha = 0.8333$$
$$\alpha = 56^\circ\ 27'$$

Thus the angular aperture is 112° 54′, indicating the tremendous light-gathering power of such a lens. This factor of α (hence the sin) can be considered as a constant for a given lens.

This fact should also be borne in mind: equation 1 applies for the space below the slide as well as for the space above. For good microscopy one should immerse with oil not only the objective but the condenser as well.

The second equation shows the relation between N.A. and resolving power.

$$d = \frac{W}{\text{N.A.}} = \frac{W}{n \sin \alpha} \qquad 2$$

Resolving power is defined as the ability of a lens to reveal two adjacent objects as separate and distinct. It is called d in equation 2. This equation in simple terms means that one can see smaller things in the microscope as the wave length gets smaller, as the N.A. gets larger, or both.

The foregoing is a brief resume of the story of microscopical optics. To some the information given is perhaps reminiscent of the classroom days. Very few, however, make use of the facts as given them then. Too often the biologist when he has a slide to examine takes out his microscope and, setting to work, either places the microscope on a desk by a north light, or perhaps in front of a glaring electric bulb. Worse still, a "substage" lamp may be used. If the light is too bright the condenser is then lowered or the iris is closed. At last, by some devious means, the illumination is

adjusted to the worker's demands, but the result of such manipulations is a complete loss of true definition. Something is seen in the field, to be sure, but what is seen may not be there at all or conversely, what is actually there frequently is not seen.

The knowing biologist selects a lamp with an iris diaphragm. Opening the iris at the condenser, he sets to work to adjust his light to his microscope. First he assures himself that the condenser is properly centered, since this is a very important point. Now he closes the lamp diaphragm to a mere pinpoint of light. Next he focuses the slide with the low-power lens. Then by manipulating the mirror the small spot of light is centered in the field. With the slide still in focus the condenser is raised or lowered until a sharp image of the light source (i.e. the pinhole opening) is brought in focus as well. Both the slide and the light should be in focus at the same time. By so doing the objective is focused on the apex of the cone of light coming from the condenser; this enables the use of the full N.A. of the objective. This operation is now repeated with the high dry lens: focusing the slide first and then getting a sharp image of the pinpoint light source by adjusting the condenser. This will have to be centered again and the same operation repeated with the oil-immersion lens. When both the slide and light source are seen in sharp focus together the iris at the lamp is slowly opened until the bright area just fills the field. For the usual oil-immersion lens the iris at the lamp will have an opening of less than a quarter inch, but this is enough.

Now the eyepiece is removed, and, while looking down the tube of the microscope, the condenser iris is closed until it can be seen just cutting into the bright spot that should nearly fill the back lens of the objective. After replacing the eyepiece the microscope is ready for use. This is not strictly critical illumination, but such a procedure is a practical approximation.

In doing this one takes advantage of all of the possibilities of N.A. and resolving power. Just enough light is entering and no more. If there should be too great a brilliancy, and this is possible even with a quarter-inch iris opening, the lamp can be moved away; or better still, a neutral filter may be used. All this may seem too time consuming to be of value. Let it be said here, however, that but little time is needed after a little practice. Having once accustomed oneself to proper methods it is felt that no one will return willfully to slipshod technique.

A lamp is shown in figure 18 which, though inexpensive, meets most requirements of good illumination. The light source is a 40-watt bulb. It is surrounded by a long asparagus can with a square of metal placed over the opening. In this metal square a hole is drilled. The hole is just

large enough for the issuing light to fill the field of the oil-immersion lens. Such a lamp, or one of similar construction, is recommended for routine observation of milk films. It is cheap, efficient, and practical.

Standard Methods suggests that to attain a given field size it should be done by "selection of oculars or by adjustment of the drawtube or

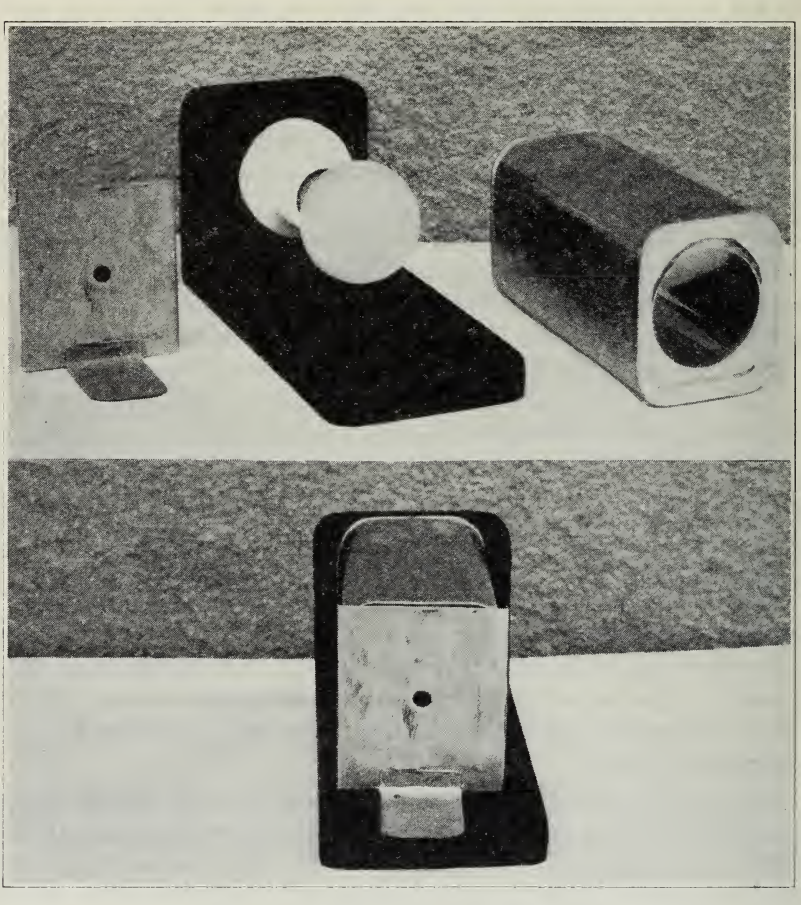


Fig. 18.—Upper: Details of the microscope lamp suggested for use with the direct count. The socket may be an integral part of the desk as in figure 8. Lower: The microscope lamp assembled; the ¼-inch opening with a 40-watt lamp gives ample light for oil-immersion objectives.

both." The field diameter desired is 0.205 millimeter and to get this the drawtube must be drawn out to some definite length. This is distinctly at variance with good microscopy. The microscope is made to work best at a certain drawtube length (160 mm for most microscopes). If this drawtube length is altered, and it must be altered if one follows the

direction given in *Standard Methods,* the image is distorted. The desired field size can be obtained by changing the ocular diaphragm. In most microscopes this is fixed, but the manufacturers can easily make one of the proper dimensions.

Provisional Methods for Other Products than Milk and for Particular Organisms.—As intimated in its early pages, this circular was written to acquaint the uninitiated with the apparatus and methods used in the bacteriological examination of milk. Careful study of the foregoing directions together with a little practice, should help one to perform the task. Only milk has been discussed. The present edition of *Standard Methods of Milk Analysis* describes methods other than for milk, as well as methods for determination of specific organisms, i.e. *Eschierichia coli* and others.

Since all these are merely variations of the basic method, detail will not be given. Anyone mastering the methods given in the fore part of this circular can with but little added practice perform the others. A word or two about them may, however, be in order.

As to the study of thermophiles, the microscope is more dependable than the plate count. As the name indicates, they are bacteria that grow best at high temperatures, where most bacteria are killed. They will not grow on special media suggested for their study any better than they will grow on the standard medium. Milk suspected of containing thermophiles will, if incubated at 140° F, give ample evidence of their presence when stained smears are made.

Although the methods suggested in *Standard Methods* for determining hemolytic bacteria are adequate, the microscope should be used along with the plating methods.

As for ice cream, the methods already outlined should be sufficient. In California the gravimetric method is followed. As butter boats sterilized in cotton-plugged test tubes are used for weighing the amount of ice cream used for plating, a supply of such boats should be purchased.

Bacterial numbers in analysis of butter are of little significance, because the cream from which butter is made is often old or has been ripened by addition of starter.The presence of yeasts and molds is a sanitary index to the methods of butter production. In sampling, slices of butter ¼ inch thick are removed from the surface of a print at a corner. For this purpose a spatula sterilized in a flame is used. Two or three grams of the freshly exposed butter are then transferred to a sterile petri dish. The butter is melted either in the incubator or over a flame. The melted butter is then plated in the usual manner, but with warmed pipettes and warm dilution water (40° C). Bacto-dehydrated whey agar is used.

It is bottled in definite quantities (100 milliliters); and, immediately before use, acid is added (4 milliliters of 5 per cent lactic acid), which decreases the pH to 3.5. This step is taken to subdue the growth of bacteria. The molds are counted after 48 hours; the yeasts after 5 days. A temperature of 30° C seems desirable, although some workers prefer 20°, and still others 25°. There is nothing "standard" in the method.

Though no definite rules of procedure will cover all the problems that arise, two things should be kept in mind in accounting for high bacterial numbers: the possibility of *contamination* and the possibility of *growth*. Obviously it is impossible to produce milk absolutely free from bacteria. The object of inspection is to keep this contamination at a minimum. If contamination, to a small extent at least, is unavoidable, then the next obvious step is to keep the contaminants from increasing in numbers—that is, to prevent growth.

The possibilities of source of contamination are legion. Unclean pipelines, cans, coolers—all may contribute their share of bacteria. In fact, no place in the plant is free from suspicion. If contamination is ruled out, then the possibility of growth should be considered. Diligent search should be made to find out whether milk is trapped in the system at places where bacteria can grow.

Always the answer to the problem will be either contamination or growth with occasionally but one exception—the cow. Let the cow ordinarily be the last consideration. Too frequently is the poor animal blamed for high counts when man himself is the real culprit.

The bacteria found in milk are generally divided into five main groups according to their action:

Acid (coagulating): sour milk; casein is precipitated.

Acid: sour milk slightly, but casein is not precipitated.

Inert: effect no visible change.

Alkali: turn milk alkaline forming "sweet curd."

Peptonizing: digest milk.

There are combinations of these as well, such as peptonizing and alkali, according to the strain of causal organism. Any bacterial change in a milk may be described as one or another (or a combination) of these type fermentations. In addition, the course of fermentation that a milk will generally follow depends on the dominance of one of these five types.

If the bacteriologist could determine the kinds of bacteria as well as the numbers, he could more easily determine the fitness of a milk for human consumption. Unfortunately, neither the plate count nor the direct count tells much more than numbers. Experience will dictate cer-

tain interpretations in this direction. Often the appearance of a colony (or colonies) on the plate or the presence of certain groups in the field of the microscope will tell a significant story, though not an infallible one.

Peptonizers, for instance, have a characteristic greenish or brick-red appearance at times. Occasionally they are large white colonies. They usually represent dirty utensils. Small pinpoint colonies may indicate growth. Long chains under the microscope also indicate growth (or, sometimes, garget), while a considerable number of large rod forms may mean dirty utensils again. Constant observation and the correlation of laboratory findings with information gathered from other sources will soon make one an efficient operator.

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